of glucose. Alanine (10mM) or arginine(20mM) were added, and the cells were incubated for 90 minutes, at which time samples were collected for assay of insulin content. The results are shown in Table 13.

Table 13

Effect of amino acids on glucose stimulated insulin secretion.

total mM glucose	control	+10 mM alanine	+20 mM arginine
1 mM	91	103	128
2 mM	108	135	134
6 mM	112	127	105
11 mM	116	117	93
16 mM	128	101	99
22 mM	115	87	94

Results: At low glucose concentrations (1, 2, and 6 mM), alanine increased insulin secretion beyond the level of glucose stimulation alone. The effect of alanine was most pronounced at 2 mM glucose, where alanine increased insulin secretion 1.25 fold over that stimulated by glucose alone. Arginine had a pronounced effect at 1 mM glucose, where arginine increased insulin secretion 1.4 fold over that stimulated by glucose alone.

EXAMPLE 21

This example demonstrates that human pancreas cells maintained in long-term culture contain immunoreactive insulin.

Human pancreas cells from Example 16, passage generation 47, were fixed and permeabilized by -20°C methanol, mounted, and stained by a standard immunochemical technique (Harlow, E. et al., 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratories) using as a primary antibody an anti-human insulin antibody raised in guinea pig from Peninsula Laboratories, Belmont, California. The secondary antibody was anti-guinea pig IgG (whole molecule) - TRITC conjugate (Rb) from Sigma (T-7153). As a negative control, in place

of the primary antibody, cells were incubated with the same primary anti-insulin antiserum which had been pre-incubated with synthetic human insulin to adsorb the anti-insulin antibodies. The cells were counterstained with Hoechst dye 33258. The immunostained cells were observed and photographed on a Zeiss IM35 microscope using a Zeiss #15 filter to illuminate the rhodamine dye labeling for insulin. The identical cell fields were observed and photographed using a Zeiss #2 filter to illuminate the Hoechst labeling of DNA in the nuclei of all cells in the field. Photographs of cell nuclei were compared with counterpart photographs of rhodamine labeled cell cytoplasms to determine how many cells in the field contained immunoreactive insulin.

Results: Controls showed no background staining. Comparison of numbers of labeled nuclei with numbers of cells immunoreactive to insulin revealed that greater than 60% of the cells in the culture contained immunoreactive insulin at different intensities of fluorescent staining.

WHAT IS CLAIMED IS:

1. A method for establishing hormone-secreting cells in vitro comprising the steps of:

a) selecting at least one cell having hormone-secreting potential from a population of similar cells having hormone-secreting potential,

b) placing said cell in an establishing medium, said establishing medium being capable of promoting the viability of said cell for at least about 13 days in vitro, and

c) maintaining the viability of at least one of said cell and the progeny of said cell for at least about 13 days in vitro.

2. The method according to claim 1 wherein,

said cells are selected to have at least two characteristics selected from the group consisting of:

(a) a smooth outer membrane,

(b) one of an approximately spherical shape and a substantially ovoid shape ,

(c) a non-granular cytoplasm, and

(d) being a member of a clump of cells numbering from about 2 to about 12 cells and being approximately homogeneous in size and shape.

3. The method according to claim 1 comprising the further step of subdividing said progeny into a plurality of cell cultures.

4. The method of claim 2 wherein,

said selection is accomplished by visual observation through a microscope, and

further comprising the step of aspiration of at least one of an individual cell and cell clumps.

5. The method of claim 1 wherein,

said method is centrifuge-free.

6. The method of claim 5 wherein,
said method is substantially enzyme-free.

7. The method of claim 1 and,
prior to step (a), placing a group of cells including said population of similar cells in a solution having substantially the same chemical composition as the biological fluid which naturally surrounded said group of cells in vivo, the concentration of said group of cells within said solution being less than or equal to the concentration of cells occurring within said biological fluid, and
prior to step (a), separating said population of cells from said group of cells in said solution by selecting cells having characteristics of viable cells and removing said cells from said solution.

8. The method of claim 7 wherein,
said step of selecting cells having characteristics of viable cells is accomplished by selecting cells having characteristics selected from the group consisting of:
(a) cells having a smooth plasma membrane,
(b) cells spreading in a monolayer on the bottom of the culture dish, and
(c) cells free from blood clots.

9. The method of one of claims 1, 2, 3, and 8 wherein,
said step (b) of claim 1 is accomplished by placing said cell in an establishing medium having an osmolarity of about 248 mOsm to about 300 mOsm.

10. The method of claim 9 wherein,
said step (b) is accomplished by placing said cell in an establishing medium further having:
(i) a basal medium comprising essential minerals, salts, vitamins, amino acids, and lipids,
(ii) a buffering system,
(iii) glutamine in the amount of about 6.35 mM to about 8.35 mM, and

(iv) at least one energy source selected from the group consisting of lactate and pyruvate.

11. The method of claim 9 wherein,
said placing step is accomplished by placing said cell in an establishing medium further having a serum in the amount of about 0.5% to about 15% of the total volume of the medium.

12. The method of claim 9 wherein,
said placing step is accomplished by placing said cell in an establishing medium further having a serum substitute in the amount of about 5% to about 15% of the total volume of the medium.

13. The method of claim 11 wherein,
said placing step is accomplished by placing said cell in an establishing medium having human serum.

14. The method of claim 13 and,
the additional step of obtaining said serum from the blood of a donor of said cell.

15. The method of claim 9 wherein,
said step (b) is accomplished by placing said cell in an establishing medium further having mammalian serum protein in the amount of about 0.5% to about 3.0% (w/v).

16. The method of claim 15 wherein,
said placing step is accomplished by placing said cell in an establishing medium having bovine serum albumin.

17. A cell culture produced according to the method of claim 9.

18. A cell culture produced according to the method of claim 9 wherein,

said population of cells is derived from a tissue selected from the group consisting of ovary, endometrium, pituitary, thyroid, and pancreas.

19. A cell culture produced according to the method of claim 9 wherein,

said population of cells is derived from a non-tumorous tissue.

20. A method for the long-term maintenance of hormone-secreting cells in vitro comprising the steps of:

a) placing at least one cell in a defined culture medium having an osmolarity of about 248 mOsm to about 300 mOsm, said cell having hormone-secreting potential and said cell being capable of proliferating in said medium to form progeny, and

b) propagating the progeny of said cell, said defined culture medium being capable of promoting the viability of at least some of the progeny of said cell.

21. The method of claim 20, and

prior to step (a), obtaining said cell from a tissue selected from the group consisting of ovary, endometrium, trophoblast, pituitary, thyroid, and pancreas.

22. The method of claim 20 wherein,

said placing step is accomplished by placing a cell in said defined culture medium which will produce progeny having potential for secretion of at least one hormone belonging to the group consisting of estrogens, progestins, follicle-stimulating hormone, luteinizing hormone, human chorionic gonadotrophin, thyroxin, glucagon, and insulin.

23. The method of claim 20 wherein,

said step (a) is accomplished by placing said cell in a culture medium further comprising:

(i) a basal medium containing essential minerals, salts, vitamins, amino acids and lipids,

(ii) a buffering system,

(iii) protein,

(iv) at least one energy source selected from the group consisting of lactate and pyruvate, and

(v) glutamine in the amount of about 6.35 mM to about 8.35 mM.

24. The method of claim 23 wherein said culture medium further comprises a serum substitute in an amount of about 5% to about 15% of the total volume of the medium.

25. The method of claim 20 wherein,

said placing step is accomplished by placing said cell in a defined culture medium having an osmolarity in the range of about 269 mOsm to about 275 mOsm.

26. A cell culture produced according to a method of one of claims 20, 21, and 22.

27. A cell culture produced according to the method of claim 20 wherein

said cells are derived from pancreas, and

said cells secrete a maintenance level of insulin.

28. A cell culture according to claim 20 wherein

said maintenance level of insulin is about 2 uIU to about 1000 uIU insulin/hour per 10^5 cells per milliliter of defined culture medium.

29. A cell culture according to claim 20 wherein

said maintenance level of insulin is about 20 uIU to about 400 uIU insulin/hour per 10^5 cells per milliliter of defined culture medium.

30. A method according to claim 20 further comprising:

subsequent to said propagating step, placing said progeny in a glucose-poor medium, and thereby

causing said cells to secrete a basal level of insulin.

31. A cell culture produced according to claim 30 wherein, said basal level of insulin is about 20 uIU to about 250 uIU insulin/hour per 1.5 million cells per milliliter of glucose-poor medium.

32. A method according to claim 27 and, after said step of placing said progeny, contacting said cells with about 0.5 mM to about 22 mM glucose.

33. A cell culture produced according to claim 32 wherein, said cells are responsive to said glucose contact to produce increased insulin secretion in an amount of about 1.2 fold to about 130 fold a basal level of insulin secretion, said basal level being in the range of about 20 uIU to about 250 uIU insulin per 1.5 million cells per milliliter of medium.

34. A cell culture according to claim 33 wherein, said response occurs over a time period comprising about 30 minutes to about 24 hours.

35. A method according to claim 30, and after said step of placing said progeny, contacting said progeny with about 2 mM to about 9 mM glucose.

36. A cell culture produced according to the method of claim 35 wherein,

said cells respond to said glucose contact to produce an increase in said insulin secretion in an amount in the range of about 1.5 to about 10 fold said basal level of insulin secretion.

37. A method according to claim 30 further comprising, subsequent to said placing step, contacting said cells with 1 mM to 6 mM glucose, thereby causing said cells to secrete an intermediate level of insulin, and

contacting said cells with an amino acid.

38. A cell culture produced according to claim 37 wherein,

said cells respond to said amino acid contact to produce an increase in insulin secretion in a range of about 1.3 to about 2.0 fold said intermediate level of insulin secretion.

39. A cell culture according to claim 38 wherein said amino acid is at least one of alanine and arginine.

40. A cell culture according to claim 39 wherein said amino acid comprises alanine in a concentration of about 10 mM.

41. A cell culture according to claim 39 wherein said amino acid comprises arginine in a concentration of about 20 mM.

42. A cell culture produced according to the method of claim 20 wherein,

said cells are derived from a non-tumorous tissue.

43. A method for obtaining viable follicular cells comprising the steps of:

a) placing in a medium at least one ovarian follicle, said follicle comprising an ovum and zona radiata cells adhering to said ovum,

b) loosening said zona radiata cells from said ovum, and

c) stripping said zona radiata cells from said ovum.

44. The method of claim 43 wherein,

step (b) is achieved by contacting said follicle with sperm.

45. The method of claim 43 wherein,

step (c) is accomplished by aspirating from said medium and expelling into said medium said follicle until separation of said zona radiata cells from said ovum is accomplished.

46. The method of claim 43 further comprising placing said cells in an establishing medium.

47. The method of claim 46 further comprising,

placing said cells in a defined culture medium having an osmolarity of about 248 to about 300 mOsm, and

propagating the progeny of said cells.

48. A method for obtaining a hormone comprising the steps of:

a) placing at least one cell in a defined culture medium in vitro, said cell having hormone-secreting potential and said cell being capable of proliferating in vitro to form progeny,

b) propagating the progeny of said cell, said culture medium being capable of promoting the viability of the progeny of said cell, said progeny secreting a quantity of hormone into said medium, and

c) isolating at least a portion of said quantity of hormone.

49. The method of claim 48, and the further step of:

contacting said cell progeny with a secretogogue selected to stimulate said secretion of said hormone.

50. The method of claim 49 wherein,

said contacting is accomplished by contacting said cell with a secretogogue selected from the group consisting of follicle stimulating hormone, luteinizing hormone, chorionic gonadotrophin, potassium ion, glucagon-like peptide-1, glucose, cAMP and chemical analogs of cAMP.

51. A method according to claim 48 and,

prior to step (a) pre-treating said cell in vivo by administering at least one of a hormone and hormone-analog drugs to a cell donor.

52. A method according to claim 51 wherein,

said pre-treating step is accomplished by pre-treating a female donor.

53. The method of claim 51 wherein,

said hormones are selected from the group consisting of follicle stimulating hormone, luteinizing hormone, chorionic gonadotrophin, and gonadotrophin releasing hormone.

54. The method of claim 53 wherein,

said step (a) is accomplished by placing a granulosa cell obtained from a preovulatory follicle in said defined culture medium.

55. A method for assaying the potency of an unknown gonadotrophin comprising the steps of:

a) providing an established cell line which secretes a known amount of a specified steroid hormone in response to contact by a specified amount of a known gonadotrophin, said known gonadotrophin having a known biopotency,

b) contacting the cells of said cell line with said unknown gonadotrophin,

c) determining the quantity of said steroid hormone secreted into the medium surrounding said cells, and

d) comparing said quantity with said known amount to determine the biopotency of said unknown gonadotrophin.

56. The method of claim 55 wherein,

said step (a) is accomplished by employing ovarian follicular cells as said established cell line.

57. The method of claim 55 wherein,

step (a) is accomplished by employing human ovarian follicular cells.

58. The method of claim 55 wherein,

said step (a) is accomplished by providing a cell line which secretes a known amount of progesterone.

59. The method of claim 55 wherein,

said step (a) is accomplished by providing a cell line which secretes a known amount of estrogen.

60. A method for determining the toxicity of a test compound comprising the steps of:

a) providing an established cell line having cells which exhibit a characterized response to a known toxin, said response being a known change in the hormone-secretion profile of said cells of said cell line,

b) contacting said cells with said test compound,

c) determining the hormone-secretion profile of said cells after step (b), and

d) comparing the hormone-secretion profile of said cells after step (b) with said known change in hormone-secretion profile to determine the relative toxicity of said test compound.

61. A cell culture comprising:

hormone-secreting cells and an establishing medium, said establishing medium comprising:

(a) a basal medium having essential minerals, salts, vitamins, amino acids, and lipids,

(b) a buffering system,

(c) an osmolarity of about 248 mOsm to about 300 mOsm, and

(d) glutamine in the amount of about 6.35 mM to about 8.35 mM.

62. The cell culture of claim 61 wherein,

said medium further comprises at least one energy source selected from the group consisting of lactate and pyruvate.

63. The cell culture of claim 61 wherein,

said medium further comprises serum in the amount of about 0.5% to about 15% of the total volume of the medium.

64. The cell culture of claim 63 wherein,

said serum comprises at least one of a human serum and a defined serum supplement.

65. A cell culture as defined in claim 61 wherein said defined medium includes a protein.

66. The cell culture of claim 65 wherein,
said medium further comprises at least one energy source selected from the group consisting of lactate and pyruvate.

67. The cell culture of claim 65 wherein,
said osmolarity is about 269 mOsm to about 275 mOsm.

68. The cell culture of claim 61 wherein,
said secreted hormone is selected from the group consisting of estrogens, progestins, follicle-stimulating hormone, luteinizing hormone, human chorionic gonadotrophin, thyroxin, and insulin.

69. The cell culture of claim 65 wherein said secreted hormone is selected from the group consisting of estrogens, progestins, follicle-stimulating hormone, luteinizing hormone, human chorionic gonadotrophin, thyroxin, glucagon and insulin.

70. An establishing medium for use in establishing hormone-secreting cells <u>in vitro</u> comprising:
(a) a basal medium having essential minerals, salts, vitamins, amino acids, and lipids,
(b) a buffering system,
(c) an osmolarity of about 248 mOsm to about 300 mOsm, and
(d) glutamine in the amount of about 6.35 mM to abut 8.35 mM.

71. A defined culture medium for use in maintaining hormone-secreting cells in long-term culture comprising:
(a) a basal medium having essential minerals, salts, vitamins, amino acids and lipids,
(b) a buffering system,
(c) protein,

(d) an osmolarity of about 248 mOsm to about 300 mOsm, and

(e) glutamine in an amount of about 6.35 mM to about 8.35 mM.

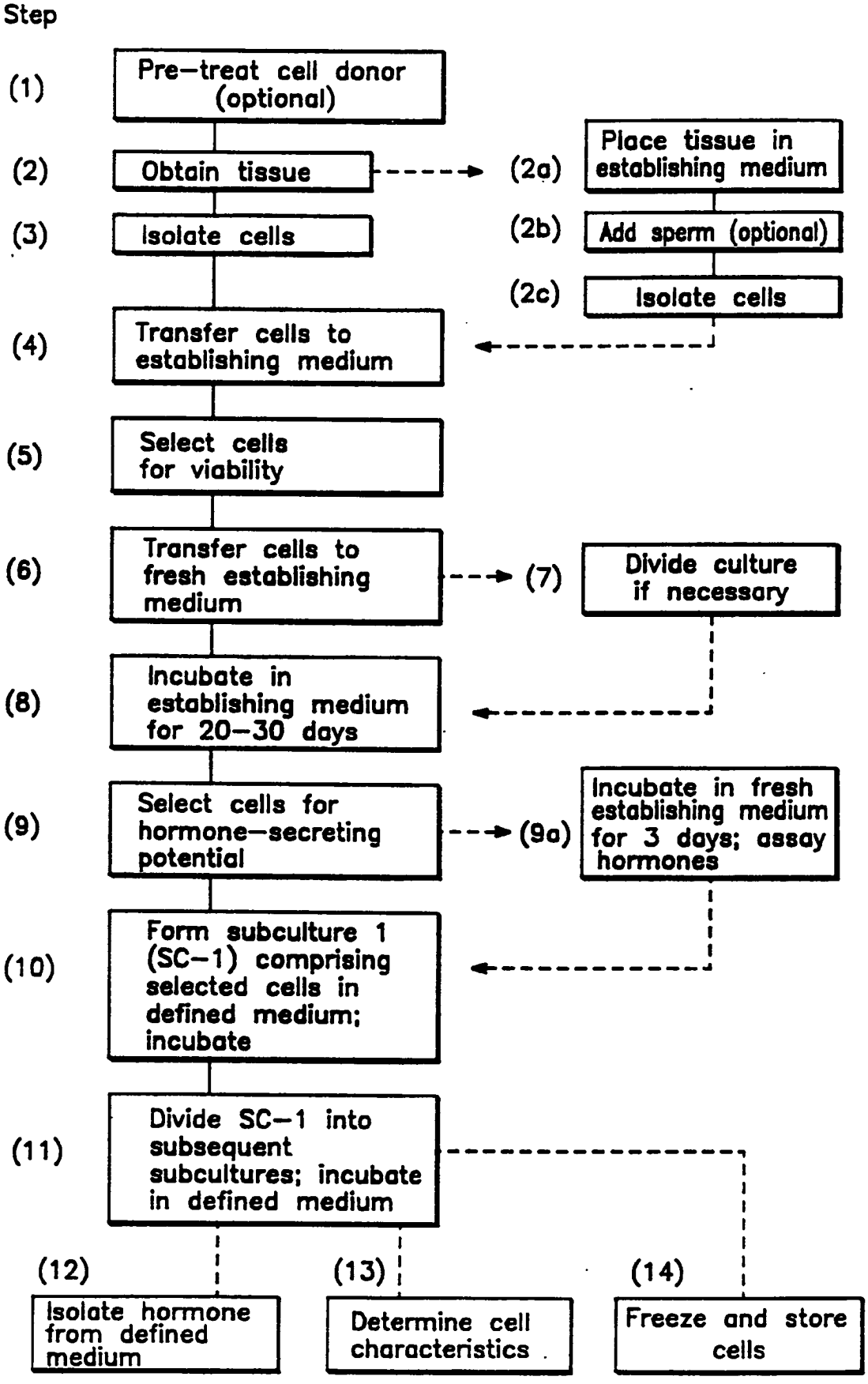

FIG.-1

FIG.—2

FIG.—3

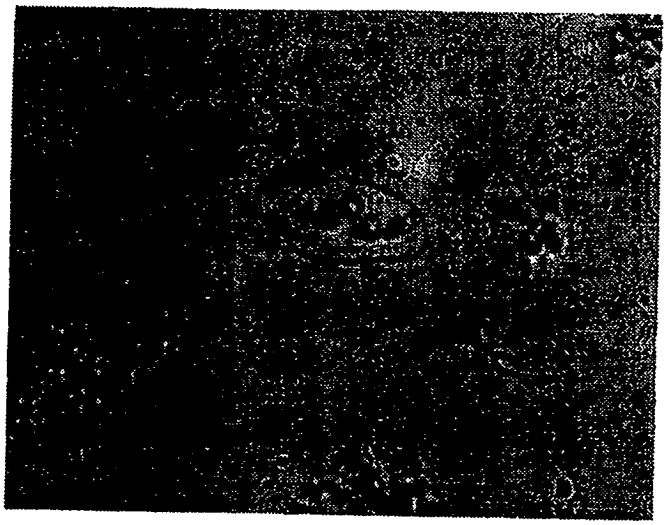

FIG.—4

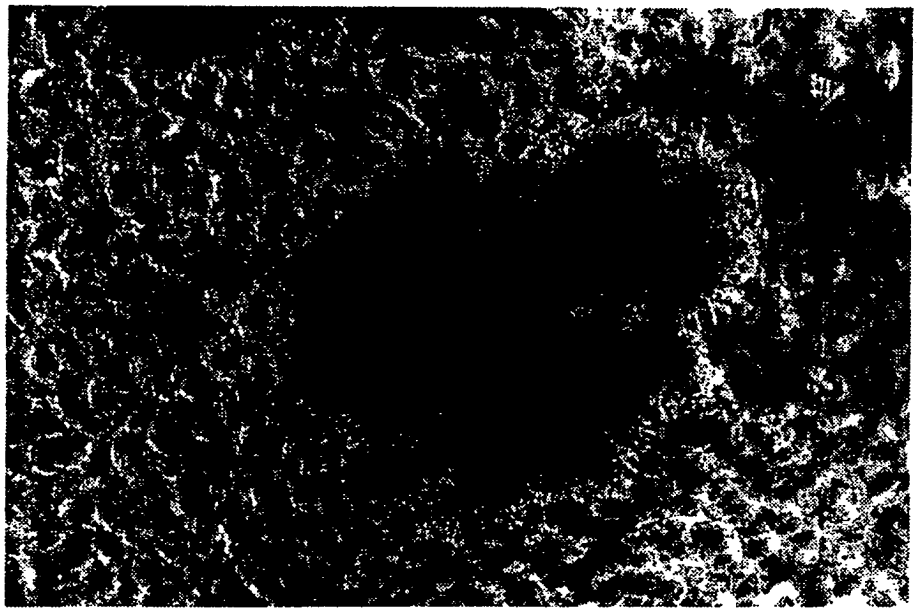

FIG.—5

FIG.—6

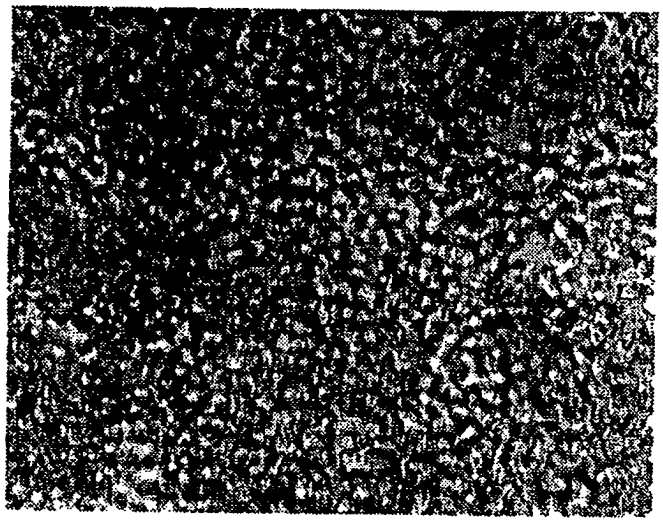

FIG.—7

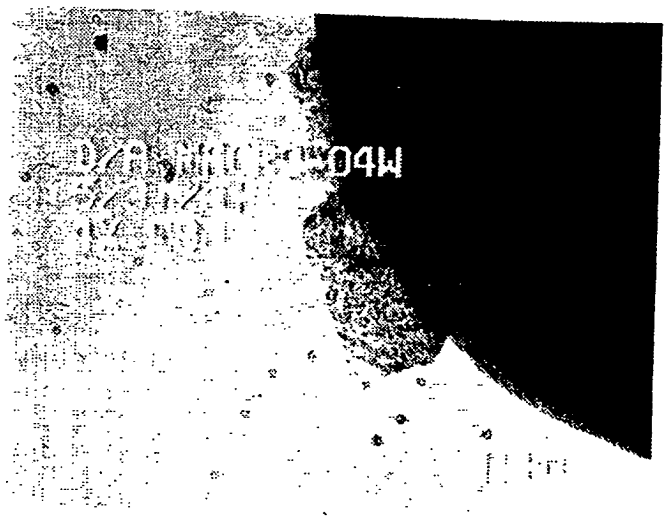

FIG.—8

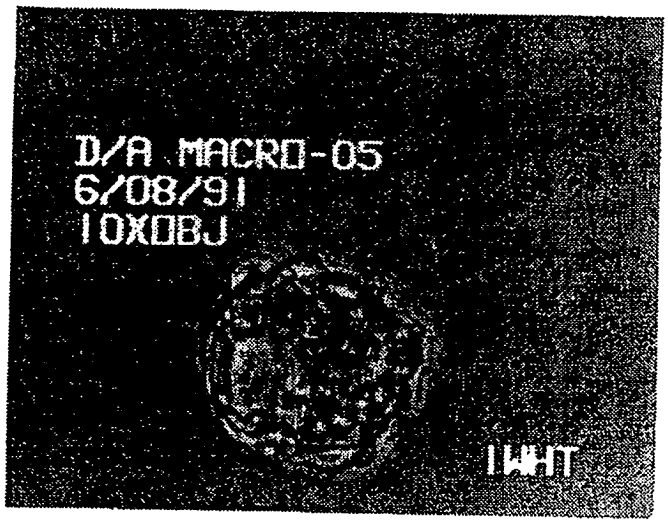

FIG.—9

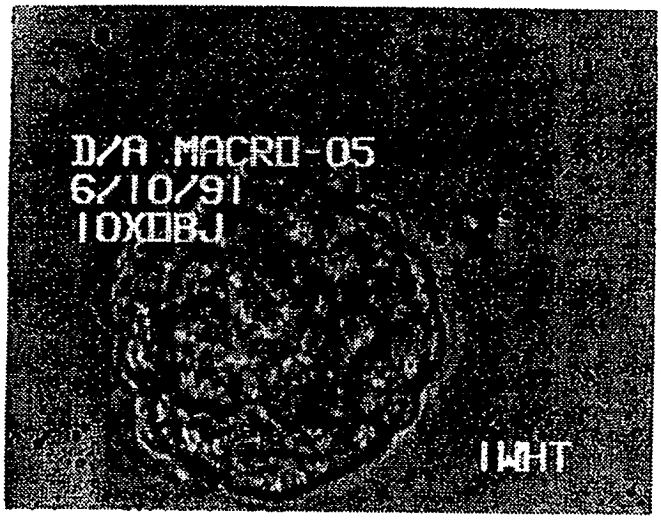

FIG.—10

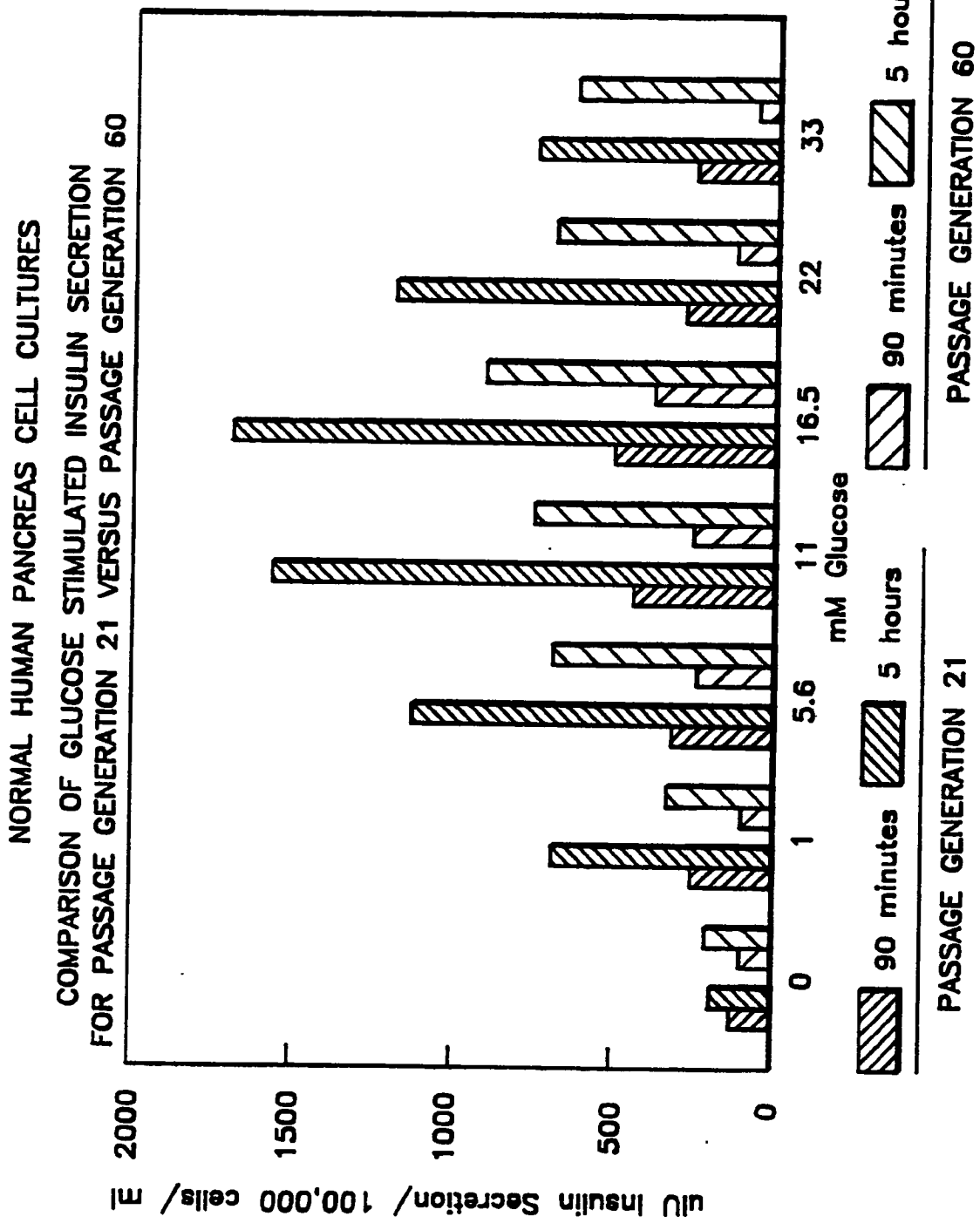
NORMAL HUMAN PANCREAS CELL CULTURES
COMPARISON OF GLUCOSE STIMULATED INSULIN SECRETION
FOR PASSAGE GENERATION 21 VERSUS PASSAGE GENERATION 60
uIU Insulin Secretion/ 100,000 cells/ ml
2000
1500
1000
500
0
0
1
5.6
11
16.5
22
33
mM Glucose
90 minutes
5 hours
PASSAGE GENERATION 21
90 minutes
5 hours
PASSAGE GENERATION 60

FIG.—11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05267

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12P 19/00
US CL :435/72.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/72.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cancer Res., Vol. 28, issued July 1968, R.A. Pattillo & G.O. Gey, "The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro", pages 1231-1236, see entire document.	20-42, 61-71
Y	J. Endocr., Vol. 113, issued 1987, K.W. Ng et al., "Isulin release from a cloned precursor beta cell line", pages 3-10, see entire document.	20-42, 48-54
X — Y	Endocrinology, Vol. 125, No. 3, issued 1989, J.L. Tilly & A.L. Johnson, "Regulation of androstenedione production by adenosine 3',5'-monophosphate and phorbol myristate acetate in ovarian thecal cells of the domestic hen", pages 1691-1699, see entire document.	43-54, 60

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search
09 SEPTEMBER 1992

Date of mailing of the international search report
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Endocrinology, Vol. 124, No. 4, issued 1989, A. Amsterdam et al., "Synergistic effect of human chorionic gonadotropin and extracellular matrix on in vitro differentiation of human granulosa cells: progesterone production and gap junction formation", pages 1956-1964, see entire document.	48-54, 61-71
Y	Endocrinology, Vol. 123, No. 4, issued 1988, J.S. Mondschein et al., "Effects of transforming growth factor-ß on the production of immunoreactive insulin-like growth factor I and progesterone and on 3H-thymidine incorporation in porcine granulosa cell cultures", pages 1970-1976, see entire document.	60-71
X — A	In Vitro Cellular & Developmental Biology, Vol. 25, No. 9, issued September 1989, R. Takaki, "Culture of pancreatic islet cells and islet hormone producing cell lines "morphological and functional integrity in culture"", pages 763-768, see entire document, especially page 763, line 40.	1-20 — 21-71